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(54) Title: HUMAN VASCULAR PROGENITOR CELLS

(57) Abstract: The invention relates to human CD34+CD31- vascular progenitor cells which are obtainable from human foetal aorta explants and which are capable of forming new vascular structures in vitro by culture in a three-dimensional collagen matrix.

Human vascular progenitor cells

The present invention relates to human vascular progenitor cells obtainable by isolation from a tissue selected from explants of human foetal aorta, human liver and human foetal heart.

If cultured in a three-dimensional collagen matrix, these cells are capable of forming new capillary-like vascular structures ex vivo.

A subject of the invention is therefore human vascular progenitor cells as defined in Claim 1.

The cells of the invention are also capable of being expanded in vitro whilst remaining in an undifferentiated form, which renders them particularly suitable for use in a series of medical applications which provide for the regeneration and/or repair of vascular structures or tissues.

The progenitor cells of the invention were first of all isolated from human foetal aorta explants in the course of experiments directed towards studying the ability of the human foetal aorta to give rise to new vascular structures in vitro. These progenitor cells were then also isolated from human foetal liver and heart.

A second subject of the invention is therefore a method of isolating vascular progenitor cells as defined in Claim 5.

In order to study the ability of the human foetal aorta to give rise to new vascular structures, aortic ring explants from 10-12 day embryos, embedded in collagen gels, (Elsdale and Bard, 1972, J. Cell Biol. 54:626-637; Nicosia and

Ottinetti, 1990, Lab. Invest. 63:115-122) were observed for 10 days.

During the first 24 hours, there were no significant changes, although a few spindly, fibroblast-like cells migrated into the matrix. Over the subsequent 24 hours, cohesive cellular cords began to sprout from the aortic rings. The number of cord-like structures was highly variable and difficult to quantify because of the complexity of the three-dimensional outgrowth and abundant fibroblast-like cells. Few cords appeared to arise from the cut edges of the explant, whereas most emerged from adventitial or intimal surfaces.

By the third day, the cords had grown haphazardly, dividing into branches and forming complex arborizing patterns. At this time, some of the newly-formed cords had regressed, whereas others were still forming and developing into capillary-like structures. Maximum cord elongation (2-3 mm) was observed after five days, after which there was rapid regression which was complete towards the end of the week. These findings were recorded in 70% of the cultures (n=15).

Another subject of the invention is therefore a method of forming new vascular structures as defined in Claim 9.

Characterization of embryonic aorta

Histological and immunohistochemical analyses were conducted to characterize the embryonic aortic tissue before culture in vitro in collagen gel.

The embryonic aorta walls consisted of an endothelial lining, a internal elastic lamina, and several layers of spindly mesenchymal cells. These cells were arranged in compact fascicles and had ultrastructural features of poorly

differentiated smooth muscle, including small bundles of myofilaments surrounded by elastic fibres. At this time of the embryonic development, vasa vasorum were absent.

Immunohistochemistry showed that only the cells lining the lumen were stained strongly for CD31 and von Willebrand factor (vWF), which confirmed their mature endothelial phenotype. None of the other cells constituting the aortic wall presented a specific immunoreactivity for these antibodies.

The staining with CD34, an antigen typically expressed in endothelial progenitor cells but also in stem cells of other origins, clearly showed that not only the lumen but also the more peripheral/para-aortic cells expressed this antigen. In a serial section of the same explant used for the CD31 analysis, CD34 seemed to be expressed by the para-aortic tissue whereas, in several sections of different rings, it seems to be constitutively and specifically expressed by the external cells of the aortic wall, suggesting that it is not, or not only, a para-aortic cell feature. The external cell layer furthermore showed a strong reactivity with antibodies for the vascular endothelial growth factor receptor 2 (VEGFR2), also known as "Flk-1", another marker of endothelial immaturity, which was also expressed by endothelial cells lining the lumen.

Characterization of vascular-like cords

The cellular phenotype of the cords and capillary tubes arising from aortic rings was investigated by light microscopy, immunohistochemistry, and electron microscopy. The outgrowths consisted of mesenchymal spindle cells, sometimes forming aggregates with central necrotic cores. Incipient formation of capillary-like structures was often

evident in areas where mesenchymal cells were densely packed. Cohesive cells with abundant cytoplasm and prominent nuclei lined the capillary-type lumen of these outgrowths. These endothelial-like cells tended to form delicate networks of long straight channels which sometimes branched at an acute angle. Immunohistochemical analysis showed that all of the cells lining the channels stained strongly for CD31 and CD34. They were also immunoreactive for vWF, though less strongly. The surrounding mesenchymal cells, which were not organized in vascular structures, were consistently negative for all of these markers.

Moreover, the cells forming the neovessels as well as the endothelial cells lining the lumen, stained strongly for Flk-1/VEGFR-2. In some rare cases, the micro-vessels seemed to originate both from the internal endothelial layer and from the periphery of the aortic section. This phenomenon was recorded in approximately 1 of 20 sections analyzed. In a very large number of histological sections, in fact, the most of neovascular proliferation occurred primarily in the outer aspects of the aortic rings whereas, in a very limited number of cases, it seemed to originate in the endothelial lining of the aortic lumen.

These observations, together with the characteristics of the untreated embryonic aorta, suggest that the neovessels arose mainly from immature endothelial precursors (CD34+/Flk1+) of the aortic external mesenchyme, i.e. that vasculogenesis rather than angiogenesis was the main process responsible for the vascular outgrowth in these cultures.

As is known, vasculogenesis in fact consists of the de novo formation of new blood vessels from undifferentiated precursors or angioblasts.

By electron microscopy studies, it was found that the outgrowth which originated from the aorta was composed of an admixture of primitive mesenchymal cells, endothelial-lined neovessels, and cells with a mixed mesenchymal/endothelial phenotype, suggesting differentiation of the mesenchyme into endothelium. The lining of the neovessels was composed of differentiated endothelial cells connected by junctional complexes. The endothelial cells exhibited a well-defined luminal/abluminal polarity and rested on a thin and discontinuous basal lamina. The endothelial cytoplasm contained abundant rough endoplasmic reticulum with focally dilated cisternae, Golgi complexes, pinocytotic vesicles, mitochondria, free ribosomes, and secondary lysosomes including osmiophilic myelin figures. Immature mesenchymal cells contained abundant glycogen, mitochondria, and bundles of microfilaments with fusiform densities which were particularly noticeable in subplasmalemmal locations. Cells with a mesenchymal/endothelial transitional phenotype tended to align to form longitudinal ordered structures and to establish junctional connections with one another. This caused the separation of newly formed luminal spaces from the surrounding cellular matrix. Cells sequestrated within the vascular lumina as a result of these morphogenetic changes lost their anchorages to the surrounding matrix and died, leaving behind cytoplasmic debris that was eventually found within the differentiated neovessels.

Isolation of CD34+ vascular progenitor cells

A cell suspension of freshly-dissected foetal aortas was used to isolate the vascular progenitor cells (CD34+CD31-) present in the aortic wall.

For this purpose, the aortas can be digested with a collagenase-dispase solution. The resulting cell suspension

was analyzed for the presence of CD34+CD31- cells by reaction with antibodies which specifically recognize these surface antigens.

For this purpose, the suspension may be incubated with magnetic CD31-antibody-coated beads to remove differentiated endothelial cells, and then with beads coated with an antibody against CD34.

This procedure demonstrated that less than 1% of the cells present in the suspension were CD31-positive (CD31+), whereas 29.5 ± 2.5% of the cells were CD34-positive (CD34+).

This result was confirmed by analysis by flow cytofluorimetry (FACS).

To better characterize these cells at the time of their isolation, some of them were plated onto dishes coated with collagen-fibronectin and cultured for a short time (24 hours) to avoid differentiation, in basal endothelial medium (EBM), supplemented with 10% foetal calf serum (FCS) and endothelial cell-growth supplement (Alessandri et al., 1998, Lab. Invest. 78:127-128). The next day, the cells were fixed and stained with CD31 and vWF antibodies, not showing any immunoreactivity.

Furthermore, to determine whether selected CD34+/CD31- cells could differentiate into mature endothelium, they were seeded and cultured for 7 to 10 days in the same conditions described above. The cells were then detached with trypsin and incubated with anti-CD31-coated beads.

As a result of this treatment, 25% ± 6.5% (mean of 5 experiments) of the initial CD34+/CD31- cell population differentiated into CD34+/CD31+ cells, which expressed the

vWF marker, thus demonstrating the formation of a more mature endothelial phenotype. Under the same culture conditions, none of the CD31-CD34- cells differentiated into CD34+/CD31+ cells or acquired specific immunoreactivity for vWF (data not shown). To further confirm the maturation process, the CD34+ cells were cultured for 2 to 3 weeks and then seeded onto Matrigel to evaluate their capability to form capillary-like tubular structures. In addition to the mature endothelial cells (CD31+) isolated from the aortic lumen and cultured under the same conditions, the CD34+/CD31+ cells were also able to form a net of capillary-like structures after incubation for 24 hours.

A further subject of the invention is therefore mature human endothelial cells as defined in Claim 8.

Discussion

Despite the recent increase in our understanding of the molecular mechanisms which regulate embryonic vasculogenesis and angiogenesis, there is still much to learn, particularly in relation to human beings. In an attempt to fill this gap, the Applicant used the aortic ring assay (Nicosia and Ottinetti, 1990) to analyze the vasculogenesis properties of 10 to 12 week-old human embryonic aortae.

In brief, the results of this study showed that, when cultured in a three-dimensional collagen matrix, human embryonic aortic explants produce an intense outgrowth of capillary structures, the in vitro formation of which resembles the in vivo physiological process of embryonic vasculogenesis. The Applicant's immunohistochemical and ultrastructural findings suggest that these outgrowths may arise from the differentiation of immature CD34+ cells and not from pre-existing endothelial cells (CD31+).

In fact, as shown by the immunohistological data, the immature CD34+ cells also express Flk-1/VEGFR-2, another marker highly expressed on the endothelial progenitor cells. These cells are localized in the more external mesenchymal layers of cells constituting the aortic wall, in strict correlation with the para-aortic tissue which, however, does not contain mature endothelial cells at this stage of development, as demonstrated by their negative staining for CD31.

As far as the Applicant is aware, the present invention describes the ex vivo formation of human microvessels by vasculogenesis for the first time. A previous study conducted by other authors on fragments of foetal human placenta blood vessels did not show this mechanism for new capillary formation.

The Applicant's data also indicate that the process of endothelial cell (EC) differentiation in human beings is different from that which occurs with the use of mouse embryonic stem cells. Indeed, in contrast with mouse stem cells, the acquisition of CD31 antigen in human vasculogenesis occurs after the maturation of CD34+ cells in culture. Furthermore, the Applicant's findings indicate that the human embryonic aorta is a rich source of CD34+/CD31- vascular progenitor cells (angioblasts) which are localized all along the external layer of the aorta mesenchyme, probably in close contact with the para-aortic tissue which, at this stage of development, does not contain mature endothelium.

The Applicant has found that 20-30% of the CD34+ cells isolated from aorta gave rise to more mature endothelium. The rest of the CD34+ cells have not been investigated. A recent study carried out using mouse embryonic stem cells

(Yashmita et al., 2000, Nature 408:92-96) suggested that endothelial and mural cells (pericytes and vascular smooth muscle) may originate from the same Flk-1+ precursors, but it is not known if this process also occurs in human beings. However, the Applicant suggests that, in the embryonic aorta, it may be possible to find and isolate primitive CD34+ Flk-1+ cells that may differentiate into various vascular phenotypes. This may be an important finding because it could lead to the isolation of vascular stem cells and a number of previously published reports have indicated the enormous potential of multipotent stem cells in clinical applications.

Given the strict correlation between the formation and the organization of the haemopoeietic/endothelial and nervous systems, the possibility of combining the transplantation of angioblasts/vascular stem cells and neural stem cells in degenerative diseases represents a very appealing approach toward improving the success of this innovative therapy. Furthermore, the vascular stem cells/angioblasts could be used to investigate the molecular mechanisms involved in human endothelial cell maturation and in vasculogenesis and the Applicant's model may therefore be valuable for vascular regeneration studies. Finally, these cells offer an important alternative for the clinical treatment of ischaemia and other vascular diseases and suggest possibilities for tissue bioengineering applications and gene therapy.

In summary, the CD34+/CD31- vascular progenitor cells of the present invention, as well as the endothelial cells obtained by differentiation of the vascular progenitor cells, may be used in a series of medical applications which may be summarized schematically as follows:

- neovascularization of ischaemic tissues, as a result of thrombotic or traumatic phenomena,

- repair of vascular damage due to traumatic phenomena or phenomena of atherosclerotic origin,

- regeneration of organs by promoting vascularization of the growing tissue (liver, mammary gland, etc.),

- transplant of medullary stem cells and those of other origin (such as neuronal stem cells) to promote establishment and growth,

- establishment of grafted bony tissue,

- in all those conditions which require establishment and growth of cells and tissues in the human organism,

- production of growth and/or trophic factors for cells of different origin and provenance,

- production of angiogenetic and/or vasculogenetic factors for therapeutic purposes in man,

- gene therapy,

- tissue bioengineering, and

- endothelization of vascular prostheses and heart valves.

EXAMPLES

Preparation of aorta explants

Permission to use human foetal material was obtained from the ethical committee of the "Carlo Besta" neurological hospital and from the "L. Mangiagalli" Gynaecology and Obstetrics Clinic of Milan. The foetal tissue was taken from therapeutically or legally aborted foetuses after signed consent by the donor, following the European Community guidelines (NECTAR Network for European CNS Transplantation and Restoration).

The human embryonic aortas (10-12 weeks) were washed carefully with PBS (phosphate buffer saline) and cleaned of para-aortic material, taking great care not to damage the aorta walls. With the use of a dissection microscope, the aortas were cut transversely with a scalpel so as to produce numerous rings about 1 mm thick. The material thus obtained was stored in DMEM (Dulbecco Modified Eagle Medium) at 4°C for no longer than 2-4 hours before use.

Assessment of the vasculogenetic properties of foetal aortas

In order to assess the ability of the foetal aorta to give rise to new vascular structures in vitro, some of the rings were cultured in a collagen solution in accordance with a method already standardized and published (Nicosia and Ottinetti, 1990, Lab. Invest. 63:115-122).

Briefly, 7 volumes of type I collagen solution (4 mg/ml) prepared from rat tail (Elsdale and Bard, 1972), on ice, was mixed with 2 volumes of 5x EBM medium (BioWhittaker, Walkersville, Maryland), and 1 volume of HEPES (0.2 M). Each well of a 12-well plate was filled with 0.7 ml of collagen solution which was left to gel in a humidified incubator at 37°C for 1 hour. One ring was placed on each gel and a further 0.5 ml of collagen solution was added to cover the material. From 1 to 2 hours after the second collagen layer

had gelled, 1 ml of EBM growth medium (Alessandri et al, 1998) was added. The plates were incubated for up to 10 days. The medium was changed every 2 days.

By means of this culture method, the Applicant has shown that foetal aorta explants possess the property of forming new cord structures which are composed of cells that express markers typical of differentiated endothelial cells (CD31, CD34, Flk-1/kDR, vWF), whilst maintaining an apparent state of morphological immaturity, as is clear from a careful ultrastructural examination (see the section on "Characterization of vascular-like cords"), thus suggesting that the vascular structures originating from the cultures of the aortic rings are vessels formed by means of a differentiational/vasculogenetic process, rather than an angiogenetic process (that is, deriving from differentiated/mature endothelial cells pre-existing in the aorta lumen).

Localization of the vascular progenitor cells in the aortic tissue

The identification of the vascular progenitor cells (angioblasts) in the aortic stroma was performed by immunocytochemical methods. With the use of a monoclonal antibody which recognizes the CD31 membrane antigen (PECAM), which is normally expressed on the differentiated mature endothelium, it was possible to show that only the cells lining the aortic lumen were positive to this antigen, whereas all of the other mesenchymal cells of the stroma were negative. However, with the use of a second antibody which recognizes the CD34 surface antigen, an antigen typically expressed in endothelial cell progenitors (CD34 also represents a marker of stem cells of other tissue origin, such as, for example the bone marrow), it was possible to

show that a considerable quantity of cells present in the peripheral area in the vicinity of the para-aortic tissue were positive to this marker. Thus, whereas the mature endothelial cells of the lumen were positive both to CD31 and to CD34, the progenitor cells/angioblasts were positive only to CD34. Moreover, the endothelial progenitor/angioblast does not express the maturation marker as Factor VIII (vWF) which, however, is typically expressed in the mature endothelium.

Isolation of vascular progenitor cells from aorta explants

Some of the rings which were not used for the evaluation in the ex vivo neovascular formation test were further broken up with scissors, after repeated washing with PBS. The fragments were washed by centrifuging (1200 rpm x 10' at 4°C) and were then transferred into a 0.25% (weight over volume) collagenase/dispase solution (Boehringer, Mannheim, Germany). The material under test was transferred to a thermostatic bath at 37°C and left with agitation for 16 hours (overnight). Upon completion of the incubation, the aortic material was completely digested. The aorta pellet thus obtained was washed with phosphate buffer saline (PBS) and resuspended in 1 ml of DMEM containing 0.2% of BSA (bovine serum albumin). The aorta cells were then counted: usually about 1.2×10^6 were obtained per aorta. Phenotype analysis by cytofluorimetry generally showed a presence of about 1-3% of CD31+CD34- cells and a percentage of about 30-40% of CD34+CD31- cells. The cell suspension was then used to isolate the vascular precursors. The isolation of the endothelial progenitor cells was performed with the use of magnetic beads treated with antibodies which recognize antigens that are present on the precursors and absent in the differentiated cells.

For this purpose, the cell suspension (about 10^6 cells) was initially incubated (30' at 4°C on a rotary stirrer) (ratio of cells/beads 1:1) with beads (10^6) treated with antibodies against CD31 (DAKO, Carpinteria, California). All of the mature endothelial cells which expressed CD31 bound to the beads and could be removed from the other cells by means of a magnet (Dynal, Oslo, Norway). It was thus possible to remove all of the CD31+ cells present from the cell suspension (the separation procedure may be repeated several times). The remaining cells were then incubated with further magnetic beads treated with antibodies which recognize CD34 or the antigen AC133 (which is also expressed on the endothelial precursors but absent on the mature endothelium). The cells which bound to the CD34 or AC133 could easily be recovered by means of a magnet. The percentage of CD34+AC133+ cells which could be recovered from about 10^6 aorta cells was about 30-40%, which percentages are similar to those quantified in the cell suspension by flow cytofluorimetry.

Isolation of the vascular progenitor cells from foetal heart and liver

After digestion of the tissues with a collagenase/dispase solution, the cell suspension obtained was filtered with filters with 10 µm porosity to remove all of the cell aggregates which were not completely broken up, whilst the individual cells in suspension were recovered and washed with PBS. The cells obtained were then incubated for 30' at 4°C with Milteni anti-AC133 magnetic micro-beads with a quantity equal to about 100 µl per 10^8 isolated cells. After incubation, all of the cells expressing the AC133 antigen (the antigen which is expressed on the endothelial precursors and absent on the mature endothelium) on their surface could be recovered with the use of a column connected to a magnet, the AC133+ cells remained caught in the column whilst the

other, AC133- cells passed through the column. The AC133+ cells were then recovered easily by detaching the column from the magnet and washing the column several times with PBS. However, not all of the AC133+ cells were a pure population of vascular progenitors since the progenitors are characterized by possessing both of the antigens AC133 and CD34 on the membrane. Once the AC133+ cells had been obtained, they were therefore incubated with magnetic beads (Dynal, Oslo, Norway) treated with antibodies which recognize CD34 (this antigen is expressed on the vascular precursors and also on the mature endothelial cells). This step enabled the AC133+CD34+ cells to be separated from the AC133+CD34- cells and an almost pure population of vascular progenitor cells thus to be obtained. The percentages of CD34-positive, AC133-positive, and CD34+AC133+ doubly positive cells recovered per 10^6 cells evaluated were 27%, 10% and 3%, respectively, for digested foetal liver and 35%, 7% and 1.3% for heart. The percentages of CD34+AC133+ cells in the AC133+ population were of the order of 30% for liver and 13% for heart.

Phenotype characterization of the endothelial progenitors

Some of the cells separated by the magnetic beads which bound CD34 or AC133 were characterized phenotypically to further check the absence of CD31 and vWF. An aliquot of purified cells (about 10^4) was seeded on glass slides treated with a collagen solution and fibronectin (as described by Alessandri et al Lab. Invest. 1998, 78:127-128) and left to incubate at 37°C for about 24 hours in a culture medium composed of EBM (endothelium basal medium, BioWhittaker, Walkersville, Maryland) containing 10% of FCS (foetal calf serum). Upon completion of the incubation, the adherent cells were fixed with 4% paraformaldehyde in PBS, pH 7.4 for 10' at room temperature. 2-3 washings with PBS (0.1% Triton-X) were

followed by a washing with a 10% guinea-pig serum medium (NGS) (Gibco, Grand Island, New York). The glass slides were then incubated with mouse anti-human CD31 monoclonal antibody or anti-human vWF (used at dilutions of 1:100 and 1:80, respectively) (purchased from Sigma, St. Louis, Missouri) for about 90' at 37°C. After two washings with PBS, the cells were incubated with a solution (1:300) of cyanine dye-labelled goat anti-mouse or anti-rabbit immunoglobulin G (IgG) (Cy2, Jackson Immunoresearch, Pennsylvania), respectively, for about 45' at room temperature. After drying of the preparation in air, the fluorescent cells were displayed and photographed with a Zeiss Axiophot-2 microscope (Zeiss, Oberkochen, Germany). Generally, after immunocytochemical analysis, less than 19% of the CD34+/AC133+ cells were CD31 positive and 0% were positive to vWF within 24 hours after seeding.

Experimental evaluation of the differentiation properties of the precursors in mature endothelial cells

Once the negativity of the Applicant's preparation of endothelial precursors to the maturation markers CD31 and vWF had been established, the cells were cultured in a medium which induced differentiation of the CD34+ progenitor cells into mature endothelial cells. The differentiation medium (M-2) was composed of the following components: MI 99 (Gibco), containing 20% of FCS, 10% of Condimed (Boehringer, Mannheim, Germany), heparin (10µg/ml) (Sigma), basic fibroblast growth factor (bFGF) (20ng/ml) (Sigma), plus vascular endothelial growth factor (VEGF) (5ng/ml) (R&D System, Inc., Minneapolis, MN). The CD34+/AC133+ cells were seeded in plastics cell-culture flasks treated with type I collagen (Calf Skin, Boehring, Mannheim, Germany) 1µg/cm^2 and bovine plasmatic fibronectin (SIGMA) 1µg/cm^2. After adhesion, the cells were cultured at 37°C in 5% CO_2 with M-2

for about 5-7 days. Upon completion of the incubation, the cells were detached from the plastics with trypsin, washed with PBS and counted. The cells were then incubated (procedure as described above) with anti-CD31 magnetic beads to isolate the mature endothelial cells. Generally, about 25-35% of the CD34+CD31- cells initially seeded differentiated into CD34+CD31+/vWF+.

The endothelial cell cultures derived by differentiation in vitro from foetal aorta vascular progenitor cells expressed some classical phenotypical and functional features of cultures of mature endothelial cells isolated both from foetal tissues and from adult human tissues. In particular, like all endothelial cells, when cultured on Matrigel (Necton-Dickinson, Bedford, Massachusetts), they were able to form capillary structures similar to human capillary vessels in vitro. This ability to form vascular-like structures in vitro is a very specific property of endothelial cells.

Expansion of endothelial progenitors

The CD34+AC133+ cells from aorta explants can be amplified in vitro by culture in a culture medium which keeps some of them in the state of immature cells. The culture medium defined as "medium-6" (M-6) is composed of the following elements: EBM medium + 10% FCS + 10% Condimed + 10% H-I (hormone mix; 400 ml of H-I are composed of: 40 ml of DMEM/F12 10X, 8 ml of 30% glucose, 6 ml of 7.5% Na_2HCO_3, 2 ml of 1M HEPES, 322 ml of sterile apyrogenic H_2O, 400 mg of apotransferrin (SIGMA), 100 mg of insulin (Sigma) dissolved in 2 ml of 0.1 N HCl, 38,64 mg of putrescine (Sigma), 40 µl of $3x10^{-3}$M selenium (Sigma), 40 µl of $2x10^{-3}$M progesterone (Sigma)).

CD34+ cells kept in M-6 retain properties of immature cells (CD34+CD31-) for at least 3 weeks of culture. After this

period, a progressive percentage (50%) of CD34+ loses this antigen and is no longer able to differentiate into mature endothelium, whereas the remaining percentage retains the ability to differentiate. Up to now, it is not known to what extent it is possible to keep the CD34+ in the undifferentiated form.

Liver and heart AC133+CD34+ cells can be amplified in culture in a manner similar to that already described for the aorta. The culture medium for the expansion of the vascular progenitor cells from liver and heart is preferably composed of medium 199 supplemented with 10% FCS, 10% Condimed (Boehringer, Mannheim) 20 ng/ml bFGF, 20µg/ml heparin, and 10% hormone mix. Many CD34+AC133+ cells kept in this medium retain properties of immature cells, remaining negative to CD31 (markers of acquisition of endothelial maturity) for several weeks. However, some acquire CD31 spontaneously and can be separated with the aid of magnetic beads treated with anti-CD31 antibodies in order to be cultured separately (for example, to develop a culture of mature endothelial cells). This ability to differentiate spontaneously into mature endothelial cells persists for at least 3-4 generations (about 4-5 weeks of culture) after which many of the initial CD34+AC133+ cells tend to lose both the markers which characterize them and the ability to differentiate into mature endothelium.

Flow cytometry analysis on digested aortas

Flow cytofluorimetry analysis was performed on the total mixed cell population obtained by collagenase-dispase digestion of the aortic fragment. About 1 x 10^5 cells were collected, stained and incubated in the dark for 30 minutes at 4°C with fluoresceinated (FITC) anti-CD34 antibody and with anti-CD31 phycoerythrinated (PE) antibody (Becton

Dickinson, San Jose, California(dilution 1:10). After two washings with PBS, the cells were analyzed by flow cytofluorimetry with the use of a FACSscan (Becton Dickinson, Mountain View, California).

Histology and Immunichemistry

Formalin-fixed tissues were included in paraffin following standard histology techniques. Four-micrometer serial sections were transferred to glass slides coated with poly-lysine and rehydrated by immersion in 100% xylene and in a series of ethanol solutions of decreasing concentration (100%, 95%, 90%, 80% and 70%). The sections were then heat-treated in a microwave cooker to enhance antigenicity and allow epitope unmasking: twice for 5 minutes each in 1mM EDTA pH8, for CD31, CD34 and vWF antigens, and three times for 4 minutes each in 0.01M citrate buffer pH6 for the Flk-1 antigen. Endogenous peroxidases were inhibited for 15 minutes at room temperature with 3% hydrogen peroxide. The samples were then blocked for 20 minutes with 20% normal blocking serum and then stained with a mixture of primary antibodies produced in mouse or rabbit and directed against the following human markers: anti-CD31 (Becton-Dickinson) diluted 1:10, 30 minutes at room temperature; anti-CD34 (Serotec, Raleigh, North Carolina) diluted 1:50, 30 minutes at 37°C, anti-vWF (Dako) diluted 1:20, 30 minutes at room temperature, and anti-Flk-1 (Santa Cruz Biotechnology, Santa Cruz, California) diluted 1:50, 45 minutes at room temperature. After repeated washings, the sections were incubated for 30 minutes with the anti-immunoglobulin antibodies of species conjugated to biotin and processed according to the avidin/biotin peroxidase complex method with kit reagents (mouse IgG and rabbit IgG Vectastain; Vector Laboratories, Burlingane, California). Peroxidase activity was shown with 3,3'-diaminobenzidine (Menarini-Biogenex, San

Ramon, California) in PBS and staining with haematoxylin-eosin. To exclude false positives produced by non-specific binding of the secondary antibody, all of the tissues were treated in the same manner with buffer substituting for the primary antibody.

Cord formation on Matrigel

270 microlitres of Matrigel (12.5 mg/ml) (Becton Dickinson, Bedford, Massachusetts) at 4°C were transferred to pre-chilled 24-well culture plates, using sterile pipette tips that had been cooled to -20°C before use. After gentle agitation to ensure a even coating, the plates were incubated for 30 minutes at 37°C to allow the Matrigel to solidify. The CD34+/CD31- cells were then seeded at a concentration of 6×10^3/well in EBM medium (Alessandri et al., 1998). Cord formation was shown after incubation for 24 hours.

Electron microscopy

Selected cultured explants were fixed in 2.5% glutaraldehyde immediately after preparation, postfixed in osmium tetroxide, embedded in Epon-Aaraldite and observed under a Zeiss CEM 902 microscope.

CLAIMS

1. Human CD34+/CD133- vascular progenitor cells which are capable of forming new vascular structures in vitro by culture in a three-dimensional collagen matrix, and which are obtainable by isolation from a tissue selected from explants of human foetal aorta, human liver and human foetal heart.

2. Cells according to Claim 1, characterized in that said isolation comprises the steps of:

a) producing a cell suspension from a tissue selected from an explant of human foetal aorta, human liver, and human foetal heart,

b) identifying within said cell suspension the cells which express the CD31 surface antigen, by reaction of cell suspension with a first antibody which binds the CD31 antigen specifically,

c) removing from the cell suspension the cells which express the CD31 antigen, which were identified in the previous step, thus obtaining a suspension of CD31- cells,

d) identifying in said suspension of CD31- cells the cells which express the CD34 surface antigen, by reaction of said CD31- cell suspension with a second antibody which binds the CD34 antigen specifically, and

e) removing from the CD31- cell suspension the cells which express the CD34 antigen, which were identified in the previous step, thus obtaining CD31-/CD34+ cells.

3. Cells according to Claim 1 or Claim 2, characterized in that they are capable of being expanded in vitro in an

undifferentiated form by culture on endothelial basal culture medium (EBM) containing, in addition, 10% foetal calf serum, 10% Condimed, and 10% hormone mix.

4. Cells according to any one of Claims 1 to 3, characterized in that they are capable of being expanded in vitro by culture on the endothelial basal culture medium (EBM) whilst remaining in an undifferentiated form for a period of at least 3 weeks.

5. A method of isolating vascular progenitor cells according to any one of Claims 1 to 4, comprising the steps of:

a) producing a cell suspension from a tissue selected from an explant of human foetal aorta, human liver, and human foetal heart,

b) identifying within said cell suspension the cells which express the CD31 surface antigen, by reaction of said cell suspension with a first antibody which binds the CD31 antigen specifically,

c) removing from the cell suspension the cells which express the CD31 antigen, which were identified in the previous step, thus obtaining a suspension of CD31- cells,

d) identifying in said suspension of CD31- cells the cells which express the CD34 surface antigen, by reaction of said CD31- cell suspension with a second antibody which binds the CD34 antigen specifically, and

e) removing from the CD31- cell suspension the cells which express the CD34 antigen, which were identified in the previous step, thus obtaining CD31-/CD34+ cells.

6. A method according to Claim 5 in which the first antibody which binds the CD1 antigen specifically is bound to a first solid substrate and the second antibody which binds the CD34 antigen specifically is bound to a second solid substrate.

7. A method for the preparation of mature CD31+vWF+ endothelial cells comprising the culture, in vitro, of progenitor cells according to any one of Claims 1 to 4 on MI 99 medium containing 20% of foetal calf serum, 10% of Condimed, 10µg/ml of heparin, 20 ng/ml of bFGF, and 5ng/ml of VEGF.

8. Mature CD31+vWF+ endothelial cells obtainable by the method according to Claim 7.

9. A method of forming new vascular structures in vitro, comprising the culture of cells according to any one of Claims 1 to 4 and 8 in a three-dimensional collagen matrix.

10. Cells according to any one of Claims 1 to 4 and 8, for use in the regeneration of vascular structures.

11. Cells according to any one of Claims 1 to 4 and 8 for use in the repair of vascular damage.

12. Cells according to any one of Claims 1 to 4 and 8 for use in the transplant of cells and/or tissues for promoting the establishment and/or growth thereof.

13. Cells according to Claim 12 for use in the transplant of medullary or neuronal stem cells.

14. Use of cells according to any one of Claims 1 to 4 and 8 in the repair of vascular damage, and/or the regeneration of vascular structures, and/or the transplant of cells and/or

tissues to favour the establishment and/or the growth thereof.

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(54) Title: HUMAN VASCULAR PROGENITOR CELLS

(57) Abstract: The invention relates to human CD34+CD31- vascular progenitor cells which are obtainable from human foetal aorta explants and which are capable of forming new vascular structures in vitro by culture in a three-dimensional collagen matrix.

INTERNATIONAL SEARCH REPORT

Interna[illegible]plication No
PCT/IT 02/00347

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, MEDLINE, EPO-Internal, PAJ, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ALESSANDRI GIULIO ET AL: "Isolation of vascular progenitors from human embryonal aorta." JOURNAL OF HUMAN VIROLOGY, vol. 4, no. 3, May 2001 (2001-05), page 146 XP001122836 2001 International Meeting of the Institute of Human Virology;Baltimore, Maryland, USA; September 09-13, 2001 ISSN: 1090-9508 abstract	1,2, 8-11,14

-/--

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 January 2003	28/01/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Noë, V

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ALESSANDRI GIOLIO ET AL: "Outgrowth of branching microvessels from human fetal aorta explants in vitro: An angiogenesis and vasculogenesis process." PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL, no. 41, March 2000 (2000-03), page 650 XP002227165 91st Annual Meeting of the American Association for Cancer Research.;San Francisco, California, USA; April 01-05, 2000, March, 2000 ISSN: 0197-016X abstract	1,9
X	GIRELLI M ET AL: "Human embryonic endothelial cells: a new tool for therapeutical approach in neurological disorders?" SOCIETY FOR NEUROSCIENCE ABSTRACTS, vol. 26, no. 1-2, 2000, pages Abstract No.-327.14, XP001109455 30th Annual Meeting of the Society of Neuroscience;New Orleans, LA, USA; November 04-09, 2000 ISSN: 0190-5295 the whole document	8,12,13
X	HEWETT P W ET AL: "Human lung microvessel endothelial cells: Isolation, culture, and characterization." MICROVASCULAR RESEARCH, vol. 46, no. 1, 1993, pages 89-102, XP008012295 ISSN: 0026-2862 abstract	8
A	MASUDA H ET AL: "Endothelial progenitor cells for regeneration." HUMAN CELL: OFFICIAL JOURNAL OF HUMAN CELL RESEARCH SOCIETY. JAPAN DEC 2000, vol. 13, no. 4, December 2000 (2000-12), pages 153-160, XP001133828 ISSN: 0914-7470 the whole document	10-12,14
P,X	ALESSANDRI GIULIO ET AL: "Human vasculogenesis ex vivo: Embryonal aorta as a tool for isolation of endothelial cell progenitors." LABORATORY INVESTIGATION, vol. 81, no. 6, June 2001 (2001-06), pages 875-885, XP002227166 ISSN: 0023-6837 the whole document	1-6,9-14

INTERNATIONAL SEARCH REPORT

Inter ›nal application No.
PCT/IT 02/00347

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claim 14 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.